In International Rubber Research and Development Board annual report for 2006. Kuala Lumpur, Malaysia: IRRDB, p. 40-55.

Annual Report on Biotechnology Group
Dr. Pascal Montoro, CIRAD

ABSTRACT

This 2006 annual report by the IRRDB biotechnology group has been expanded to cover operations being conducted on *Hevea* in organizations other than rubber research institutes. The organizations involved are BGRICAS in China (Beijing Gene Research Institute of the Chinese Academy of Sciences), IRD and UBP-INRA in France, UKM in Malaysia, the universities of Mahidol (MU) and Kasetsart (KU) in Thailand, and the University of Santa Cruz in Brazil (UESC). Alongside the disciplines usually covered, strategies combining several biotechnologies are proposed and a major surge in transgenesis and genomics has led to new functions being identified and analysed.

In vitro culture research is still very active. Work has been resumed on microcuttings. The low multiplication rates for RRIC and RRISL clones have led researchers at RRISL to rejuvenate the clones by microbudding embryos onto young 1-month-old seedling rootstocks. CIRAD, IBRIEC and IRRI are currently studying the feasibility of propagating rootstock clones by microcuttings. Somatic embryogenesis remains the preferred technique for mass propagation of rubber trees on their own roots, along with the development of transgenesis. Although some Sri Lankan and Malaysian clones (RRIM 2020) remain recalcitrant, routine production of thousands of plants has been achieved by CATAS, CIRAD and Michelin. Over several years, CIRAD has been developing a technique for the cyropreservation of embryogenic callus which is now being routinely used to conserve lines with high embryogenic potential.

Plants genetically modified for endogenous functions or through foreign gene insertion are now regularly produced in *Hevea.* GFP (Green Fluorescent Protein) expression has been achieved at CIRAD to monitor the evolution of transgenic lines and eventually do away with antibiotic-based selection. Work is under way at CATAS and CIRAD to improve tolerance of cold (HbCBF1 transcription factor) and oxidative stress (CuZnSOD) respectively. A gene involved in rubber tree growth has been introduced into transgenic plants at MRB, along with several foreign genes such as those encoding HP (human protamine), PHB (bioplastic), and the ScFv4715 antibody.

Molecular physiology work is focusing on latex production and resistance to diseases. Beforehand, an interesting latex RNA extraction technique at ambient temperature was published in *J.B.B. Methods*. Sugar loading depends on transporters for which several genes have been isolated by the CATAS and UBP-INRA teams. Some researchers from MRB have discovered that the mevalonate pathway was probably not the only pathway for IPP (isopentenyl pyrophosphate) production. The DXP/MEP pathways would seem to be an alternative occurring in plasts. Six isoforms of DXP Synthase have been isolated and are undergoing characterization. Interestingly, the possible involvement of Frey-Wyssling particles in IPP synthesis is proposed through this pathway. V-PPase (Vacuole pyrophosphatase) has been located on the membranes of rubber particles and lutoids by researchers at CATAS. That enzyme would seem to catalyse the degradation of a by-product of rubber biosynthesis, pyrophosphate acid. Following the demonstration of an

activity destabilizing the rubber particles in bark extracts, analyses at MRB led to the discovery of a polymeric phenolic compound that would seem to play a crucial role in that phenomenon. Far upstream, studies on the biosynthesis and regulation of ethylene and jasmonate are being carried out at CATAS, CIRAD and IRRI to describe the molecular mechanisms controlled by those hormones affecting latex production and triggering TPD. Lastly, the HbMyb1 transcription factor involved in cell apoptosis regulation is being investigated at CATAS to determine its involvement in TPD.

Among the recent developments in functional genomics, worth mentioning is the generation of nucleic sequence libraries for expressed sequence tags (EST) in latex (30,000 EST at CATAS, 20,000 EST at MRB), but also a few thousand EST from bark (IRD, MU, CIRAD) or even from the leaves of clones that are susceptible or resistant to diseases (CIRAD, IBRIEC and UESC). MRB, in collaboration with UKM has released its data into the public domain so that the scientific community can benefit from them (http://genome.ukm.my/nrestdb). A project for a Consortium to sequence all the genes expressed in the rubber tree and develop a DNA chip will be proposed by the Biotechnology Group at upcoming IRRDB meetings. The purpose of the project is to pool resources to analyse the complete set of genes involved in development and defence against abiotic and biotic stress in the rubber tree. Although still very costly, proteomics also seem to be a fundamental approach for identifying the different cell functions. CATAS, in collaboration with BGRICA, is currently setting in place technical support for the analysis of all the latex proteins, particularly those involved in natural rubber biosynthesis. Given the evolution of these technologies, IRRDB intends to organize training and a Workshop on these subjects in 2008. The availability of these powerful tools is a decisive challenge for future advances in different areas of research designed to improve the rubber tree.

In terms of plant pathology, research using biotechnologies is primarily focusing on leaf diseases caused by *Microcyclus ulei* and *Corynespora cassiicola*. SALB is being studied as part of collaboration between CIRAD and Michelin, who are proposing that producing countries assess 13 SALB-resistant clones, which have been in quarantine at CIRAD since 2005. *Corynespora* control is looking into the diversity of toxins produced by the fungus, cassiicolin. Initial work undertaken at CIRAD shows that two strains, CCP and BCA3, isolated in the Philippines and Cameroon respectively, produce a protein of the same molecular weight. Microsequencing of the toxin from CCP is a glycosilated protein of 27 amino acids behaving like a host-specific toxin. MRB and CIRAD are working together to produce transgenic plants that are resistant to CLFD through the expression of an antibody raised against cassiicolin. A gene encoding that antibody has been isolated. The 800 pb of that gene comprise the fragments V_L and V_H, of 350 and 400 pb respectively. Characterization of a homologue of Hev b 4 lecithinase is in progress at MRB to develop a 3D model involving the structure of the N-glycan complexes.

Molecular markers are being used in multiple applications, such as germplasm characterization (CATAS, RRII, etc.), clone identification (CIRAD, RRISL), mapping (several families of crosses at CATAS, CIRAD, MRB, RRIT), etc. In addition to the neutral markers used at MRB to screen genotypes resistant to CLFD, some molecular markers of the *resistance gone analog* type (RGA) are being developed at RRISL from conserved motifs of resistance genes. At RRIT and CIRAD, a programme is attempting

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to identify QTL of growth, production and stress tolerance. For example, a QTL linked to trunk circumference has apparently been identified on chromosome 3. Marker-assisted selection is being considered.

To conclude, the Biotechnology Group will organise a Workshop on November 11, 2007 in Siam Reap, Cambodia. The purpose of this meeting is to draw up full details of an International Consortium for the development of functional genomics tools in the rubber tree. Directors of Rubber Research Institute and Corresponding members of the Biotechnology Group will be the main promoters of this initiative. The assignment of corresponding members for each IRRDB member country is required for such international scientific exchanges.

CHINA

CRRI (CATAS)

1. Basic molecular biology research techniques in *Hevea*

1) Preparation techniques for nucleic acid and protein samples

Nucleic acid (RNA and DNA) and protein samples from latex, bark and leaf of rubber tree are necessary materials for molecular biology research. The preparation methods for some parts of the samples have been established and improved. Latex is the main material of molecular biology research of rubber tree. It is necessary to use liquid nitrogen during the latex collection process using the former latex RNA extraction method. And latex shall be stored at -70℃. Thus the expenditure and input of labour increase in the experiments. It will be inconvenient when samples are taken at remote place. Recently a procedure has been established in CRRI which is suitable for latex collection, storage, long distance and long time transport and high quality RNA extraction without using liquid nitrogen and low temperature storage. Some parts of the research results have been published in *J Biochem Biophys Methods* and *Plant Physiology Communications*.

2) cDNA library construction

High quality cDNA library construction techniques of latex, bark and leaf of rubber tree have been mastered. The basis for high flux gene expression analysis has been established.

2. Functional genes cloning in *Hevea*

In recent years, the genes cloned first by the researchers in our institute and analyzed for the function include:

1) *HbMyb1* gene: It's an transcription factor gene. It was thought that this gene is a negative regulatory factor which mediates cell apoptosis. TPD (Tapping panel dryness) may be a syndrome of cell apoptosis. Some parts of the research results

have been published in *Plant Mol Biol* and *J of Plant Physiol and Mol Biol.* More systematic research results have been submitted to *Plant J.*

2) ***V-PPase*** gene in *Hevea.* The Vacuole pyrophosphatase (V-PPase) gene is located on the membrames of rubber particle and lutoid-body , and catalyze the degradation of pyrophosphatic acid, which is the by-product of rubber biosynthesis. And this gene is an important regulatory factor of rubber biosynthesis. The research results are going to be published.

3) *COI1*、*AOS and ALO* genes in *Hevea.* These are genes relative to jasmonic acid biosynthesis, regulation and signal transduction, which regulate rubber biosynthesis. The research results are going to be published.

4) *HbSUTs* gene: sucrose transport protein gene in *Hevea.* At present the whole length cDNA clones of 6 members of this gene family have been obtained. The regulation of expression of this kind of genes maybe has relation to sucrose provision efficiency in the laticifers in *Hevea.* So they are candidate genes that control rubber biosynthesis. Some parts of research results are going to be published in *Chinese Journal of Tropical Crops.*

5) *HbCBFs* gene: candidate genes correlated with cold resistance in *Hevea.* Preliminary function characterization of *HbCBF1* gene has been accomplished in transgenic tobacco plants and transgenic *Arabidopsis thaliana* plants. It has been verified that increasing its transcription can increase the accumulation of substance correlated with cold resistance, so enhance the cold resistance. Some parts of the research results have been published in *Chinese Journal of Tropical Crops.*

6) 67Kda storage protein gene in bark of rubber tree: The product of this gene has insect-resisting activity. So it is a candidate gene for protecting tapping panel of rubber tree. The research results are going to be published.

3. Functional genomics studies

Latex of rubber tree can be separated for three main parts: rubber particles (the organelle in which rubber biosynthesis takes place), C supernatant fluid of latex (the site in which the synthesis of precursors of rubber biosynthesis takes place), and components at the bottom (mainly lutoid-body, determining the stability of latex). In recent years CRRI has initiate high flux functional genomics research (including large scale cDNA sequencing, cDNA micro-array technique, 2D-PAGE and high performance liquid chromatography combining mass spectrometry analysis, etc) to study the molecular mechanism of the regulation of latex production and drainage in proteomics and transcriptomics, TPD occurrence, stress resistance and high efficiency utilization of nutrition. At present, protein samples from different components of latex can be prepared, 2D-PAGE separation and mass spectrum characterization technique have been mastered. About 30,000 EST of laticifer gene expression spectrum have been constructed. Membrame protein data bank of rubber particle has been preliminarily established. Some parts of the

research results have been published in *Chinese Journal of Tropical Crops* and IRRDB conference collected works.

4. Molecular mark assistant breeding

RAPD、ISSR、SSR、AFLP molecular mark technique systems have been established. Genetic diversity of the germplasm resource in *Hevea* has been analyzed by RAPD、ISSR、SSR methods. Average gene heterozygosis analysis shows that the genetic diversity of wild germplasm is higher than that of cultivar germplasm. The resemblant level within cultivars is higher. So the genetic basis is narrow, and it is expected to be expanded. During the work on selection of yield mark in *Hevea*, the analysis toward RRIM600×PR107 separation group has been carried out, and 2 candidate fragments relative to low yield have been obtained. Now they have been transferred to SCAR mark, and Southern analysis is being carried on. Besides, fingerprint maps of rubber tree are being constructed to characterize different clones by using SSR mark now. The analysis on cold resistance trait difference has been carried on by using cDNA-AFLP technique to acquire marks correlated with cold resistance. Some parts of the research results have been published in *Chinese Journal of Tropical Crops* and IRRDB conference collected works.

5. Tissue culture and genetic transformation

Tissue culture and plant regeneration systems from anther, inner integument and cotyledon of rubber tree have been established. In 2006 and 2007 more than 1000 vitroplants have been obtained from somatic embryos of different clones. Some progression has been achieved on cryopreservation of embryogenic callus from anther, some calli can survive and proliferate after thawing. Embryogenic cell suspension culture is being carried on.

The *HbCBF1* gene relative to cold resistance has been transformed into some somatic embryos mediated by *Agrobacterium tumefaciens.*

6. Latex genomics and proteomics research platform

CRRI collaborates with Beijing Gene Research Institute of Chinese Academy of Sciences, and has preliminarily established latex genomics and proteomics research technique platform.

Parts of the publications:

An Ze-wei, Huang Hua-sun (2005). A method for genomic DNA extraction from leaves of rubber tree (*Hevea brasiliensis* Muell. Arg.). Plant Physiology Communications, 41(4), 513-515

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detected by RAPDs and ISSRs. Journal of tropical and subtopical botany, 13(3): 246-252

Chen Shoucai, Peng Shiqing, Huang Guixiu, Wu Kunxin, Fu Xianghui, Chen Zhangquan. (2002). Association of decreased expression of a Myb transcription factor with the TPD (tapping panel dryness) syndrome in *Hevea brasiliensis*. *Plant Mol. Biol.* 51(1):51-58

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Duan CF, Nie ZY, Li Y, Zeng RZ. Proteomics Analysis of the Membrane-bound Proteins on the Rubber Particle in *Hevea brasiliensis*. *Vietnam* IRRDB Conference, 2006

Duan Cuifang, Nie Zhiyi, Li Yu, Zeng Rizhong (2006). Establishment of 2D-PAGE electrophoresis system and preliminary mass spectrum analysis of membrane- bound proteins on rubber particle in *Hevea brasiliensis*. Journal of Tropical Crops, 27(2): 22-29

Huang TD, Li WG, et al. Micropropagation of shoot apex and shoot stem with axils of cotyledon of *Hevea brasiliensis*. China IRRDB Conference, 2004

Huang TD, Li WG, Huang HS, Li Z. Somatic embryogenesis and plant regeneration from cotyledon explants of *Hevea brasiliensis*. India IRRDB Conference, 2005

Peng Shi-qing, Fu Xiang-hui, Wu Kun-xin, Cheng Shou-cai (2003). Structural analysis and expression of *HbMyb1* gene associated with tapping panel dryness of *Hevea brasiliensis*. Journal of Plant Physiology and Molecular Biology, 29(2): 147-152

Sun Ai-Hua, Li Zhe, Huang Tian-Dai (2006). Anther Culture in *Hevea brasiliensis*. Plant Physiology Communications. 42 (4): 785-789

Tang CR, Qi JY, Li HP, et al. (2007). A convenient and efficient protocol for isolating high-quality RNA from latex of *Hevea brasiliensis* (para rubber tree). *J Biochem Biophys Methods* (in press, available on line: doi:10.1016/j.jbbm.2007.04.002) .

Yang Jianghua, Huang Debao, Liu Shujing, Tang Chaorong（2007）. Cloning and sequence analysis of 6 sucrose transport protein gene in *Hevea brasiliensis*. Chinese Journal of Tropical Crops(accepted)

Zhang Fucheng, Deng Zhi, Zhang Yunxia, Chen Shoucai (2006). Cloning, sequence analysis and expression vector construction of 5' regulation region for key enzyme gene of natural rubber biosynthesis from *Hevea brasiliensis*. Chinese Journal of Tropical Crops, 27(3): 40-45

Zhang YX, Chen SC, Peng SQ (2004).Cloning of a gene that regulates the expression of the TPD associated gene HbMyb1 by yeast one hybrid. China IRRDB Conference, 2005

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FRANCE

CIRAD, IRD, INRA, Michelin

1. Somatic embryogenesis (Ludovic Lardet)

An alternative process for long term somatic embryogenesis using primary somatic embryo explants has been performed on clone PB 260. This new process was assessed on clone PB 217 showing a very low embryogenic capacity of primary compact callus. As a consequence a few primary somatic embryos were available for the experiment on secondary somatic embryogenesis. Nevertheless, we have obtained some interesting friable calluses which are maintained in culture. A better proliferation of these calluses is needed for the establishment of new embryogenic lines with this clone. This year, several thousand integument explants have been introduce with the aim to improve embryogenic capacity of primary compact calluses.

2. Assessment of new varietal types from biotechnology (Marc-Philippe Carron)

Research on Biotechnology provided different processes and data from field which now gave evidence for new varietal type ready for application: rootstock clones, whole clones on their own roots and juvenile type budded clones (figure 1).

- ✓ **Rootstock clones development** is in progress through CIRAD – IRIEC partnership (France – Indonesia). Microcutting from young seedling was mastered at the beginning of 90s and field assessments recently confirmed the good behaviour of the trees, especially regarding the root system.

- ✓ **Whole clones on their own roots**: Primary somatic embryogenesis from selected clones was also performed at the beginning of 90s and about ten clones were regenerated. This process does not allow mass propagation, but rejuvenation of the mature clones. This qualitative profit can be exploited for the micropropagation of whole clones on their own roots, as emblings used to be reactive for multiplication through microcutting process. And field assessments recently confirmed the superiority of such trees for growth and rubber yield compared to conventional budded clones.

- ✓ **Juvenile type budded clones** are in progress at CIRAD – MICHELIN team. The superiority of such material on the conventional mature budded clones was emphasized from 1940 by Dutch team. At the end of the last century, Chinese team, then French one took opportunity of rejuvenation through primary somatic embryogenesis to obtain juvenile type budded clones from about ten selected clones. Once again, field assessments confirmed the superiority of such trees for growth and rubber yield compared to conventional budded clones. The first step for clonal rejuvenation needs specialists but spreading of juvenile type budded clones will be close to the conventional process.

Otherwise, mass propagation through maintained somatic embryogenesis and genetic transformation, still in the research phase, prepare for other prospective outcomes.

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3. Propagation of transformed material

Transgenic lines have shown a decrease of regeneration potential compared to original untransformed line. Nevertheless, two hundred plantlets have been regenerated from seven transformed embryogenic lines. Expression of the GUS reporter gene will be assessed from two way propagated plantlets, grafting and micropropagation. One part of the transgenic plantlets have been acclimatized, while first attempts for micropropagation of *in vitro* plantlets are being realised with the other part.

4. Corynespora cassiicola and the determinism of host-specificity

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Corynespora cassiicola can be pathogenic for a wide range of plants species: more than 300 host plants are recorded for *Corynespora cassiicola* in the SBML Fungus-Host

Distributions database (http://nt.arsgrin.gov/fungaldatabases/fungushost/fungushost.cfm). However, each *C. cassiicola* isolate has a selective host-range. In rubber tree, each isolate is not only selectively virulent but also selectively aggressive, displaying a range of symptoms of various intensities depending on the clone (1).

We have purified cassiicolin, the toxin produced by a *Corynespora cassiicola* isolate from Philippines (CCP) (2). It is a 27-aminoacids glycosylated protein which behaves like a typical Host-Selective Toxin (HST): the purified toxin alone is able to induce the disease symptoms and shares the same host-range as the fungal strain that produces it (1,3).

However the determinism of host-selectivity is not understood yet. Are there qualitative and/or quantitative differences at the toxin level that could explain the diversity of the strains in terms of aggressiveness? To try answering this question, we have attempted the purification of cassiicolin from three other strains of various aggressiveness and geographical origin. The toxin could be purified from BCA3, a strain of medium severity isolated in Cameroun, but not from other strains with low aggressiveness on the sensitive clone PB260, due probably to the scarcity of toxin production. Both CCP and BCA3 toxins had exactly the same molecular weight, as measured by mass spectrometry, suggesting that they could be identical. However, this could not be verified by NMR analysis because the quantity of purified BCA3 toxin available was too low. We have recently cloned the gene encoding cassiicolin. This opens the way for an analysis of the cassiicolin gene diversity and regulation among the *Corynespora cassiicola* population. This is a prerequisite before considering the diversity of the plant resistance factors that may also contribute to the host-specificity.

(1) Breton, F., Sanier, C., and d'Auzac, J. (2000) Role of cassiicolin, a host-selective toxin, in pathogenicity of *Corynespora cassiicola*, causal agent of a leaf fall disease of Hevea. *J. Rubber Res. 3*, 115-128.

(2) de Lamotte, F., Duviau, M. P., Sanier, C., Thai, R., Poncet, J., Bieysse, D., Breton, F., and Pujade-Renaud, V. (2007) Purification and characterization of cassiicolin, the toxin produced by Corynespora cassiicola, causal agent of the leaf fall disease of rubber tree. *J Chromatogr B Analyt Technol Biomed Life Sci 849*, 357-62.

(3) Barthe, P., Pujade-Renaud, V., Breton, F., Gargani, D., Thai, R., Roumestand, C., and de Lamotte, F. (2007) Structural analysis of cassiicolin, a host-selective protein toxin from Corynespora cassiicola. *J Mol Biol 367*, 89-101.

5. Sugar transporters of *Hevea brasiliensis* laticiferous cells: physiological and molecular characterizations in relation with the rubber production

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Sucrose is known to be a precursor of the latex biosynthesis in laticiferous cells, and therefore it can play a key role in the rubber production in *Hevea brasiliensis*. Because laticiferous cells are photosynthetically inactive "sinks", they must import sugar to meet its high carbon and energy demands. Several circumstantial evidences assume that the sugar loading into laticiferous cells is an active mechanism, probably mediated by a H^+/sugar symporter. However, no cDNA sugar transporter has so far been identified and characterized in laticiferous cells. The main goal of our team is to give, for the first time, an insight in the molecular mechanism of sugar loading into laticiferous cells and in the potential role of these sugar transporters in the rubber production. To this end, different but complementary strategies (pattern expression, heterologous expression, *in situ*-localization....) will be used.

Briefly, laticiferous cells–derived cDNA library was screened and several sugar transporters have been identified, including sucrose transporters, hexose transporters and polyol transporters. Their expression pattern has been monitored on latex and bark tissues of two hevea clones (PB217 and PB260), using real time RT-PCR. The first data indicate that some sugar transporters are significantly up-regulated by ethylene treatment. Moreover, this ethylene-induced regulation of sugar transporters is tissue-specific and depends on the clone studied. A detailed characterization of the most interesting sugar transporters is under way in our laboratory.

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INDONESIA
IBRIEC, IRRI

Indonesian Biotechnology Team is focusing on the establishment of microcutting technique transferred from CIRAD for the provision of rootstocks clones of *Hevea*

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brasiliensis. Report on the progress of research implementation is reported by Dr. Nurhaimi Haris as following.

1. Propagation of *Hevea* rootstock clones in Indoneia
Nurhaimi-Haris, Sumarmadji, Radite Tistama, Sumaryono, MP Carron

To achieve the optimum yield of scion clones in rubber tree (*Hevea brasiliensis*), high quality of rootstock clones should be available. The provision of e such kind of plant materials could be achieved through the use of microcutting technique, developed by CIRAD team. Formal research collaboration between Indonesian Biotechnology Research Institute for estate Crops (IBRIEC), Indonesian Rubber Research Institute (IRRI) and CIRAD had been started to apply this technique. In 2005, preparation and selection of plant genotypes which will be used as mother plants in microcutting had been done at Sungei Putih (IRRI). As much as 100 genotypes had been selected from 2 ha seedling garden, consisting approximately 43.000 seedlings GT1, PB 260 and RRIM 600. Each selected genotypes had been propagated to create 10 copies/genotype and used as a source of explants for microcutting. Currently the plant materials were distributed and planted in green houses at Sungei Putih (IRRI), Bogor (IBRIEC) and Montpellier (CIRAD). By using the plant materials growth in Taman Kencana greenhouse (IBRIEC), the first primary culture had been done in February 2006 and the last primary culture in December 2006. During this period 2.722 explants from 86 genotypes had been introduced in 32 experiments. At the beginning, the percentage of contamination at the end of primary culture quite high, around 52%, however with some effort the level of contamination could to be decreased to 23%. In parallel, by decreased contamination level, growing healthy explants were increased from 20% to 80% at the end of primary culture. Mean multiplication rate is more than 200% for 20 genotypes, out of 86 in trials (25%), at the end of the 3rd subculture (one to seven repetitions per genotype). From these, 15 best genotypes, regarding the multiplication rate, were selected for further multiplication to achieve the aim of the project.

Other research activity being conducted at IBRIEC and IRRI in Sembawa is on the molecular analyses of responses of Hevea clones to ethylene stimulation. The team is consisting of Dr. Tetty Chaidamsari and Dr. Asmini Budiani of IBRIEC and Dr. Kuswan Hadi of IRRI at Sembawa, South Sumatra.

MALAYSIA
Malaysian Rubber Board

1. Rubber crop production

RUBBER BIOSYNTHESIS
Alternate rubber biosynthesis pathway

In recent years, the plastidic DXP (2-C-methyl-D-erythritol 4-phosphate) or MEP pathway has been regarded as an alternate pathway of isopentenyl pyrophosphate (IPP) production rubber biosynthesis in the rubber tree. Since the DXP/MEP pathway operates in plastids, the possible involvement of the Frey-Wyssling (FW) particles in IPP synthesis for rubber biosynthesis was explored.

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Preliminary investigations into the possible DXP/MEP pathway were performed using Moir's Zone D. The addition of Zone 4 to radiolabelled pyruvate in the presence of C-serum significantly increased incorporation of the label into rubber. In order to prove that the FW particles (in Moir's Zone D)was involved in the formation of new rubber molecules via the MEP pathway, enzyme inhibitors were introduced to the RBA. Mevilonin was used to inhibit HMG-CoA reductase in order to block the production of mevalonate and thus, new rubber formation would only take place via the MEP pathway. Fosmidomycin (or Phosphomycin) was used separately to specifically inhibit the DXP reductoisomerase, a key enzyme in the MEP pathway in order to allow formation of new rubber molecules to occur via the mevalonate pathway. Each inhibitor, when added individually to the reaction mixture, did not reduce significantly the conversion of pyruvate into isoprene. However, combinations of Mevilonin with Fosmidomycin resulted in inhibition of the synthesis of rubber from pyruvate.

Cloning of *Hevea* DXPS cDNA

IPP, the common intermediate of isoprenoid biosynthesis, may be synthesized either through the cytosolic mevalonate (MVA) pathway or the plastidic non-mevalonate (MEP) pathway. The first step of the MEP pathway is catalyzed by the enzyme, DXP synthase. A *Hevea* EST for the DXPS enzyme was identified from the MRB latex EST collection in 2005 and characterized by complete sequencing. This EST clone is 1004 bp in length but is truncated at the 5' end. To clone the full-length *Hevea* DXPS cDNA, two primer sequences were designed for 5' RACE amplification: one from the 3' end of the DXPS EST and the other from the 5' end. The 5' RACE products obtained were either 437 or 438 bp in length and DNA alignment revealed six DXPS variants sharing more than 99% identity. More upstream sequences will be obtained by designing new primers for further 5' RACE amplification.

PHYSIOLOGY OF LATEX FLOW

Analysis of Hevea *bark extract for rubber particles destabilising activity*

Further investigation on rubber particles coagulation activity of bark sap fractions revealed that the brownish retentate fraction obtained from 100 kDa spin columns to be the most active component. The retentate fraction on SDS-PAGE gave a thick smearing along the lane when stained with Coomassie Blue R250, with no distinct bands. The flow through of the spin column contained a protein at circa 40-50 kDa, presumably peroxidase which appeared to be most abundant protein in *Hevea* bark sap.

In a related experiment, 10% polyvinylpolypyrrolidone (PVPP) and polyethylene glycol (PEG) 4500 were added separately to *Hevea* bark sap; both PVPP and PEG are known to bind tannin. In a reaction which occurred within minutes, PVPP and PEG precipitated out the brownish coloration in the bark sap. The bark sap (clear) was then added to rubber particles suspension. Since no destabilisation/coagulation was observed. the brownish substance in the bark sap (presumably phenolics/tannin) could play a crucial role in rubber particles destabilisation. Experiments performed with rubber particles and

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bark extract showed that the phenolics/tannin in the bark extract is able to precipitate proteins that were detached from zone 1 and zone 2 rubber particles, thus suggesting that the destabilisation of rubber particles may be mediated by interaction between phenolics/tannin in the bark extract and (rubber particles) surface proteins.

To date, the identity of the brownish pigment/substance remains unclear; the only proposition that points towards polyphenols/tannin was its ability to bind PVP and PEG. An attempt was made to elucidate the properties of the brownish pigment in bark extract by separation through SEP-PAK C18 cartridges according to the manufacturer's instruction. Thin layer chromatography (TLC) on cellulose acetate coated glass plates was performed on the crude bark extract, and the brownish pigment, tannic acid served as standard in this experiment. It was observed that only tannic acid migrated in the solvent while the spots corresponding to the crude bark extract and the brownish pigment remained close to the origin. This showed that the *Hevea* bark active fraction could be a polymeric phenolic compound.

TISSUE CULTURE AND GENETIC TRANSFORMATION

Tissue culture of RRIM 2020

Tissue culture in *Hevea* is clone specific, therefore media which works for clone GL1 will not work for RRIM 2020. A tissue culture system for RRIM 2020 was initiated to complement the genetic transformation work which aim to generate *Hevea* trees resistant to *Corynespora* infection. RRIM 2020 was chosen because it is a clone susceptible to *Corynespora* infection. Experiments with different media performed in 2006 resulted in ten embryoids.

Hevea *transformation*

Transformation experiments using various gene constructs continued this year.

- The human Protamine cDNA (hP) gene construct transformation in *Agrobacterium* GV2260 (HP:pTok47) has resulted in one plantlet.

- Transformation using the girthing gene construct GV2260 (pLGMR.GA20: pToK47) generated four putative transgenic plantlets for GL 1, but two plantlets did not survive in soil.

- The bioplastic phb gene construct transformation has generated four putative transgenic plantlets.

- An effective gene promoter would facilitate expression of the recombinant protein in transgenic *Hevea*. *Hevea* genetic transformation using the hevein promoter construct driving the antibody gene ScFv4715 resulted in 16 putative transgenic plants that were established in soil and subsequently disbudded, resulting in 77 vegetative plants.

In International Rubber Research and Development Board annual report for 2006. Kuala Lumpur, Malaysia: IRRDB, p. 40-55.

RUBBER GENOMICS: EXPRESSED SEQUENCE TAGS

Latex ESTs: Expansion of MRB Rubber EST Collection

The MRB EST Collection currently consists of 10,000 latex ESTs and details may be viewed at the NREST Database (http://genome.ukm.my/nrestdb/) constructed in 2005. The NREST Database has been accepted into the Molecular Biology Database Collection on the Nucleic Acids Research Journal (announcement in January, 2007). Further expansion of the MRB rubber EST collection (in collaboration with the Malaysia Genome Institute at UKM-MTDC, Bangi) begun early 2006 using funds provided by the MRB Board. This resulted in a total of 20,000 latex ESTs generated by MRB to date.

Genomics of rubber biosynthesis

Genomic analysis of rubber biosynthesis in latex was undertaken to investigate the expression of genes that encode enzymes and proteins of the mevalonate (MVA) and non-mevalonate (DXP/MEP) pathways. The first method utilized EST frequency of thirteen biosynthesis-related genes while a second method using quantitative PCR analyzed the expression of these genes at a transcriptome-wide level. In both profiles, REF and SRPP were distinctly more abundant than the rest. Highest expression of REF and SRPP suggests a constitutive production of these rubber particle proteins in the laticifer to meet the 30-50% rubber content of latex.

Bioinformatics analysis of REF and SRPP sequences

The nine members of the REF/SRPP protein family account for 12% of the latex EST collection and have been named as the Rubber Particle Membrane Protein (RPMP) family. In latex protein preparations, proteins of other molecular weights have been observed in SDS-PAGE gels depending on the method used. Immunoblots of such extracts showed that REF and SRPP antibodies detected additional but less abundant proteins with molecular weights 12 and 19 kDa which may be accounted for by some of the new RPMP isoforms. Multiple alignment of nine RPMP isoforms show that they are very highly similar. A prediction of membrane spanning regions of the nine protein isoforms showed that only 5 of the proteins, including the first clones of REF and SRPP, contain hydrophobic regions suggesting trans-membrane regions. This suggests that the nine RPMPs have different degrees of association with the rubber particle membrane and this determines how easily they may be dislodged using different methods of preparation.

Comparative sequence analysis of RPMP genes between homologues in other families of the plant kingdom indicated that the RPMP gene family is not as unique to rubber as anticipated hitherto. Association with stress-related proteins suggests a dual role played by this protein family in the rubber tree i.e. stress response and rubber biosynthesis while in non-latex-bearing species of the plant kingdom, RPMP homologues show a predominant function in stress responses.

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MOLECULAR PHYTOPATHOLOGY

Three dimensional protein modelling of Hev b 4 lecithinase homologue

The complete cDNA and protein sequences as well as the mass-spectrometry analysis of the glycans of Hev b 4 lecithinase homologue are available. A detailed analyses of the N-glycan moiety of Hev b 4 revealed presence of complex type N-glycans with xylose and core α1,3-linked fucose at positions N84, N99, N142, N224, and N256; the complex type glycans have been attributed to IgE binding in earlier studies. These information were put together to generate a preliminary homology-based 3D model of lecithinase homologue. The validated model would then include the exact glycan structures derived from earlier mass-spectrometry analysis.

Transgenic rubber tree resistant to Corynespora

The first phase of the MRB-CIRAD collaborative project to develop transgenic rubber trees resistant to *Corynespora cassiicola* involved construction of an anti-cassiicolin scFv phage library to be carried out as a collaborative effort with Universiti Kebangsaan Malaysia. A second phage library was constructed mainly to increase chances of isolating anti-cassiicolin specific scFv. The presence of scFv was confirmed by PCR amplification with primers specific to scFv that resulted a 0.8 kb fragment, and restriction digest with *Bst* O1 that results in a 350 bp V_L and a 400 bp V_H fragment. The recombinant anti-cassicolin scFv proteins will be over-expressed from the selected clones for use in an *in vitro* bioassay to validate cassiicolin-deactivating ability of the recombinant proteins prior to selection of the clones for *Hevea* genetic transformation.

HEVEA GENETIC LINKAGE MAPPING

The generation of AFLP markers for the genetic linkage map continued with most of the effort directed towards the analysis gene markers of the parental clones, PB 5/51 and IAN 873, and RRIM 937 x RRIM600.

Mapping population of PB 5/51 X IAN 873 and RRIM 937 x RRIM60

Fourty five and thirty one out of 106 primer pair combinations were use to generate AFLP markers in family PB 5/51 x IAN 873 and RRIM 937xRRIM600 respectively. The markers generated were analysed using the JoinMap mapping program. Table 1 summarizes the data analysis of the polymorphic markers in these families.

PB 5/51	Marker	276
	LOD 7.0	18 groups
IAN 873	Marker	259
	LOD 7.0	15 groups
RRIM 937	Marker	242
	LOD 5.0	18 groups
RRIM 600	Marker	154
	LOD 6.0	18 groups

Table 1 Grouping of markers at LOD scores 5.0 to 7.0.

The data were analysed three times with JoinMap to ensure the most suitable LOD score for each map constructed. The target of having 700 markers per map cannot be achieved with only one enzyme combination, in this case was *EcoRI/MseI*. To increase the number of AFLP markers, other enzyme combinations will be used. Such enzyme combinations are of *PstI/MseI* and *HindIII/MseI*.

Disease Screening of PB 5/51 X IAN 873 with Corynespora cassiicola

Screening the KT mapping population to assist in detecting QTLs for resistance to *C. cassiicola* is currently underway. Previously, only 44 plants out of 86 were screened with isolate Race 2, CLN 16. Currently, the rest of the population is undergoing the screening process. Isolate Race 1, CSB 16 was found to be non-viable, thus it will be replaced by CBPP which is a Race 1 isolate.

Study of Gene Expression using the Differential Display Technique

Five plants that were recorded to be susceptible to CLN 16 (mean disease score of 0.0 to 0.5) and five plants resistant to the same fungus (mean disease score of 2.4 to 3.18) were disbudded and replicated (10-15 plants each) and placed in a greenhouse. These plants were then sprayed with CLN 16 spores in an environmentally controlled chamber after which leaves were sampled at 0 hr (control), 4, 8 up to 72 hours. RNAs will be extracted from resistant and susceptible plants and then bulked for differential display analysis.

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Database Submission

NRESTdb (= Natural Rubber Expressed Sequence Tag Database) was officially registered with the Molecular Biology Database Collection 2007 of the Nucleic Acids Research Journal. Ref: Nucleic Acids Research Vol. 35, Database Issue.

THAILAND

RRIT-DOA, Kasetsart University, Cirad Thailand

Genetic mapping and QTL approach towards growth and latex production

Genetic maping and QTL approach are developed in Thailand by Rrit-Doa, Kasetsart University and Cirad, with a view to better understanding the genetic determinism of growth, latex production and resistance to the tapping stress. This research aims at developping Markers-Assisted Selection in rubber.

A genetic linkage map of the full-sib family RRIM600 x PB217 was carried out from 2002 to 2005. A saturated consensus map was achieved, based on the genotyping of 334 progenies with a total of 445 molecular genetic markers, including 247 microsatellite and 198 AFLP markers distributed over the 18 linkage groups corresponding to the 18 chromosomes of the haploid genome of the rubber tree. The total length of the haploid genome was found of 2075 centi-Morgans, and the average genetic distance between 2 markers was of 4.86 centi-Morgans. The maximum distance found between two markers was of 38 centi-Morgans.

Phenotyping is currently being developed in a field trial planted in June 2002 at Crrc/Rrit-Doa. Tapping has just begun in June 2007, at 5-year old. QTL detection has been applied so far to growth parameters and some other traits (defoliation/refoliation, abundance of fruits, and susceptibility to die-back). The morphological variability between progenies was assessed qualitatively by visual scorings. The main visual distinction is based on the relative importance of the trunk as related to the total biomass of the tree. The second visual distinction is based on the density of ground coverage by the canopy. The girth of the trunks appears to be a good indicator of the total biomass of the trees, and an easily measurable trait. Along time, the share of the trunk and of the leaves, as compared to the total biomass, is reduced whereas the share of the branches is increased. One highly significant QTL related with the growth in girth of the trunk was identified. It explains more than 20 % of the genetic variation among progenies. Its expression as considered only during the dry seasons periods seems to be reinforced, although not significantly because growth during the dry season is very slow. This might indicate that the QTL reflects the response of the trees to water stress. The genetic marker associated to this QTL (A312, located on linkage group g3) might be used as a very early selection criteria within this family for identifying the progenies most adapted to this ecological site.

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International Rubber Research and Development Board

Annual Report for 2006

Secretariat 260, Jalan Ampang, 50450 Kuala Lumpur

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International Rubber Research and Development Board (IRRDB)

Overall Aims and Objectives

The IRRDB is a voluntary association of national organizations (Member Institutes) committed to research and development on natural rubber. The countries in which these Institutes are located cover 96 per cent of world natural rubber production. The IRRDB conducts its business through a Board, which is responsible for all matters affecting the IRRDB; a Committee of Directors and Chief Executives, whose function is to examine and discuss technical matters and to make recommendations to the Board on such matters; and a Finance Committee.

Activities of the IRRDB cover (i) exchange of ideas between staff of Member Institutes at meetings organized by the IRRDB, (ii) activities initiated by the IRRDB's Specialized Groups, (iii) training, and (iv) major projects initiated by the IRRDB and executed by one or more Member Institutes working together, in some cases with external funding. These projects constitute the IRRDB's international research programme.

There are ten Specialized Groups, each operation being coordinated by a Liaison Officer: (i) Plant Breeding, (ii) Plant Protection, (iii) Physiology, (iv) Technology and End Uses, (v) Socio-Economic matters, (vi) Agronomy, (vii) Biotechnology, (viii) Exploitation Technology, (ix) Environment and (x) Transfer of Technology.

A copy of the IRRDB Constitution is available from the Secretariat *(see below).*

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